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RESEARCH ARTICLE

Optimization of physical parameters for cellulase production by *Fomitopsis meliae* under Solid state fermentation

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Abstract

Cellulose, a major component of the most abundant lignocellulosic biomass on the Earth, can be broken down by the enzyme cellulases into simple sugars, which can then be converted into biofuel and other value-added products. Cellulases are in high demand due to their extensive applications in various industries. Brown rot fungi, a type of wood-rotting fungi, efficiently degrade the polysaccharide (cellulose and hemicellulose) present in the wood. In the present study, we cultivated *Fomitopsis meliae* (Underw.) Gilb., a facultative brown rot fungus, both under submerged fermentation (SmF) and solid state fermentation (SSF) on wheat bran, and quantified the yield of cellulases (CMCase, FPase and β-glucosidase). Cellulases were produced in higher quantity under SSF. Various physical parameters for SSF were optimized through One-factor-at-a-time (OFAT) approach, and their best level for higher cellulase production by *F. meliae* were found as: fermentation temperature - 32–36 °C, initial substrate pH – 4.0, substrate moisture ratio - 1:3, inoculum age - 4–6 days old culture, inoculum dose - 4 culture discs, substrate quantity – 5.0 g, and substrate size 0.85–2.00 mm. The fungus consistently produced very high quantity of CMCase followed by β-glucosidase than FPase.

Keywords: Brown rot fungus, CMCase, FPase, β-glucosidase, Submerged fermentation, Media optimization

1. Introduction

Cellulose, a major component of lignocellulose, is the most abundant renewable biomass on the Earth. It can be broken down into simple sugars, which can then be converted into biofuel and various other value-added products. Cellulase enzymes that hydrolyze cellulose, comprises three distinct enzymes: endoglucanase, exoglucanase, and β-glucosidase. These enzymes work synergistically to completely break down cellulose into glucose. Cellulases are considered the third most important industrial enzyme due to its extensive applications in various industries such as textiles, paper and pulp, food and feed, bioethanol, organic acid production, laundry detergents, and waste management (Sajith et al., 2016).

Cellulases are produced by several microorganisms, including fungi, bacteria, and actinomycetes. Fungi are particularly suitable for cellulase production because they produce large amounts of extracellular cellulases in fermentation media. Several fungal species from the phyla Chytridiomycota, Ascomycota, and Basidiomycota are recognized as efficient cellulose degraders (Fliegerova et al., 2015; Payne et al., 2015). Among them, ascomycetes such as Trichoderma reesei and *Aspergillus niger* have been employed for commercial cellulase production (Saini et al., 2015). Additionally, a group of basidiomycetes known as Brown rot fungi (BRF) have very efficient cellulolytic systems that selectively remove cellulose and hemicelluloses from wood while leaving the surrounding lignin intact (Eriksson et al., 1990; Arantes and Goodell, 2014). They degrade wood in a two-step process that involves generation of highly reactive oxygen species (ROS) to attack and open up the lignocellulosic complex, and secretion of cellulases and hemicellulases. The composition, mode of action, and catalytic efficiency of the cellulase complex vary among *T. reesei*, *A. niger*, and BRF (Payne et al., 2015; Hamid et al., 2015; Yang et al., 2011; Yoon and Kim, 2005; Valášková and Baldrian, 2006; Veloz et al., 2020; Kipping et al., 2024). The search for novel fungi suited for various industrial and biotechnological applications is ongoing.

The yield of cellulases depends on the fermentation technique used, whether Submerged Fermentation (SmF) or Solid State Fermentation (SSF), and the growth conditions provided. In SmF, microorganisms grow in a free-flowing liquid with the substrate dispersed within it, whereas SSF is carried out on a solid substrate moist enough to support microbial growth. SmF offers easy handling and better management of growth conditions (Singh et al., 2007), while SSF is cost-effective, provides a suitable environment for filamentous fungi, and requires less downstream processing. It has been reported that SSF provides higher enzyme yield than SmF from the same strain of microorganisms (Prévot et al., 2013).

In this study, we evaluated cellulase production by a brown rot facultative parasite fungus, Fomitopsis meliae, in SmF and SSF. Based on the results, we attempted to enhance enzyme production under SSF by optimizing various physiological parameters.

2. Materials and methods

2.1 Chemicals and raw material

Potato dextrose agar (PDA), carboxymethylcellulose sodium salt (CMC), congo red were purchased from HiMedia. Dinitrosalicylic acid (DNSA) was obtained from Sisco Research Laboratories (SRL), and p-nitophenyl-β-D-glucopyranoside (pNPG) was procured from Sigma-Aldrich. All other salts used in the experiments were of analytical grade. The wheat bran for fermentation process was sourced from the local market. It was thoroughly washed and then dried either in sunlight or at 60 °C in the hot air oven till constant weight was achieved.

2.2 Fungal sample

Fomitopsis meliae (Underw.) Gilb. (Family Fomitopsidaceae, Order Polyporales, Class Agaricomycetes, Phylum Basidiomycota) collected from the Botanical Garden of Rajiv Gandhi University, was cultured

on Potato Dextrose Agar (PDA) and maintained at 4 °C. The rDNA sequence consisting of 628 nucleotide (18S ribosomal RNA gene, partial sequence; ITS1, 5.8S ribosomal RNA gene, and ITS2, complete sequence; and 28S ribosomal RNA gene, partial sequence) was deposited in GenBank (Accession No. PP818976).

2.3 Qualitative screening for cellulases

Fungal culture was inoculated on PDA containing 2% CMC. After 72 hours, the cultured plates were flooded with 0.2% congo red and left for 10 minutes at room temperature (25–30 °C). Subsequently, the congo red was discarded, and the plates were washed with distilled water, and destained with 1M NaCl for 5 minutes, repeating the process 2–3 times till a halo zone appeared around the culture. To stabilize and inhibit hydrolysis, the plate was flooded with 1M HCl (Teather and wood, 1982).

2.4 Cellulase production under SmF

Wheat bran (1%) as carbon source, 2% malt extract and 50 ml of mineral salt solution (MSS) were thoroughly mixed in a 250 ml Erlenmeyer flask and autoclaved. Subsequently, 4 fungal discs, crushed using mortar and pestle, were inoculated to the substrate. The flasks were then placed in a rotary shaker at 150 rpm and incubated at 30 °C for 20 days. Enzymes were extracted daily starting from day 3 onwards by filtering the fungal culture through muslin cloth. The enzyme extract was then centrifuged at 10000 rpm at 4 °C for 12 minutes, and the collected supernatant used for enzyme assay. Experiments were conducted in triplicate.

2.5 Cellulase production under SSF

Wheat bran (5g), as sole carbon source, was placed in a 250 ml Erlenmeyer flask, and 15 ml of MSS (containing $(NH_4)_2SO_4$, KH_2PO_4 and $MgSO_4.7H_2O$ each at 0.5 g/L) was added to moisten the substrate. After through mixing, the mixture was autoclaved at 121°C for 15 minutes. Once cooled, the substrate was inoculated with 4 mycelial discs each measuring 8 mm in size, taken from a 10-day old pure culture. The flasks were then incubated at 30 °C for 18 days in a BOD incubator. Enzymes were extracted daily starting from day 3 onwards.

For enzymes extraction, 0.5M citrate phosphate buffer (CPB; pH 5) was added to the flask. After thorough mixing, the flasks were kept in an incubator shaker at 150 rpm for 45 minutes at room temperature. The solution was filtered through muslin cloth and centrifuged at 10000 rpm for 12 minutes at 4 °C. The supernatant was collected and assayed for crude enzyme activity. Experiments were performed in triplicate.

2.6 Enzyme assay

For endoglucanase activity (CMCase), 0.5 ml of diluted crude enzyme was added to the test tube containing 0.5 ml CMC (substrate) prepared in sodium citrate buffer (pH 4.8) and incubated at 50 °C for 30 minutes following the method of Ghose (1987). The reducing sugar was measured using the DNSA method (Miller, 1959) and read at 540 nm. One unit of enzyme activity was defined as the amount of enzyme required to release 1 µmole of the reducing sugar per minute.

Filter Paper Assay (FPA) was performed for total cellulase activity following the method of Ghose (1987). In a test tube, 0.5 ml diluted crude enzyme was added to 1 ml of sodium citrate buffer (pH 4.8). A strip of Whatman filter paper (1 X 6 cm, 50 mg) was immersed and incubated at 50 °C for 60 minutes. The released reducing sugar were measured by adding DNSA (Miller, 1959) and recording the absorbance at 540 nm. One filter paper unit (FPU) was defined as the amount of enzyme required to release 1 µmole of reducing sugar from the filter paper per minute.

β-glucosidase activity was assayed according to the method described by Wood and Bhat (1988). In a test tube, 1 ml of pNPG prepared in sodium acetate buffer (pH 4.8), 1.8 ml of sodium acetate buffer and 200 µl of diluted crude enzyme were mixed and incubated at 50 °C for 30 minutes. Reaction was completed by adding 4 ml NaOH-glycine buffer (pH 10.8) and absorbance was measured at 430 nm. One unit of enzyme activity was defined as the the amount of enzyme required to liberate 1 µmole of p-nitrophenol from pNPG per minute.

2.7 Optimization of physiological parameters for SSF

Various physical factors (Table 1) were evaluated by using One-factor-at-a-time (OFAT) approach to optimize SSF for enzyme production. Crude enzyme was extracted on the 17th day and assayed for enzyme activity.

Table 1. List of the physical parameters optimized for SSF

Exp. No.	Physical parameters	Level	Fixed level of other physical parameters
1	Temp. (°C)	28–40	SQ 5 g, pH 5, SMR 1:3, ID 4 discs, IA 10 days
2	pH	4–8	Same as experiment 1, Temp. 32°
3	Substrate moisture ratio (SMR)	1:1–1:5	Same as experiment 2, pH 4
4	Inoculum age (IA; days)	4–10	Same as experiment 3, SMR 1:3
5	Inoculum dose (ID; number of culture disc)	1–7	Same as experiment 4, IA 6 days
6	Substrate quantity (SQ; g)	5–20	Same as experiment 5, ID 4 discs
7	Substrate size (mm)	0.15–2.00	Same as experiment 6, SQ 5 g

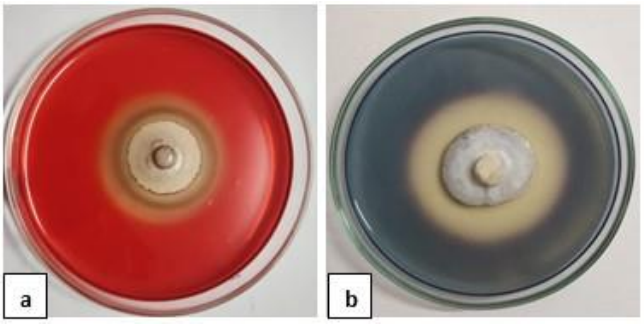

Figure 1: Colony of *F. meliae* showing zone of hydrolysis up on treatment with (a) Congo red + NaCl, (b) Congo red + NaCl + HCl

3. Result

Formation of a prominent halo zone around the culture *Fomitopsis meliae* indicated that it is a cellulase-producing fungus. The stabilization and inhibition of hydrolytic activity were indicated by change in media colour from red to blue after applying HCl.

3.1 Time course study on cellulase production

3.1.1 Under SmF: Incubation of *F. meliae* for cellulase production under SmF was carried out for 20 days, and quantification of enzyme production was done from 3rd day onwards. CMCase was typically produced in greater amounts than FPase and β-glucosidase (Figure 2). The Production of CMCase and FPase increased from day 8, reached its maximum between days 9 and 11, peaked again on day 13, and showed a minor rise on day 19. High CMCase production consistently correlated with increased FPase production. In contrast, β-glucosidase production gradually and slowly increased from day 8, peaking only on day 19, which corresponded with the third peak of CMCase and FPase production.

3.1.2 Under SSF: Incubation of *F. meliae* for cellulase production under SSF was carried out for 18 days, and quantification of enzyme production was done from 3rd day onwards. Compared to SmF, the incremental rise in production of all the three enzymes occurred earlier, and their production level was higher (Figure 3). CMCase was always consistently produced in much greater amount than other two enzymes from day 5 onwards. However, unlike SmF, FPase production from day 7 onward was always much lower than that of β-glucosidase. CMCase production showed two peaks: one between day 8 and 12, and a larger one between from day 15 and 17. FPase production did not show any distinct peaks, with yield fluctuating

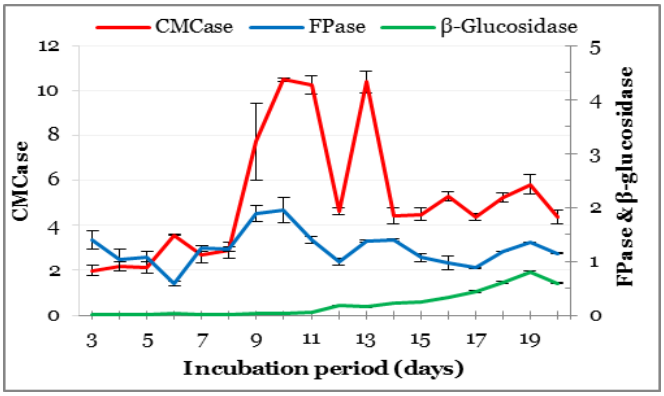

Figure 2: Time course study on cellulase production (IU/ml) under SmF by *F. meliae* (values are Mean±SEM)

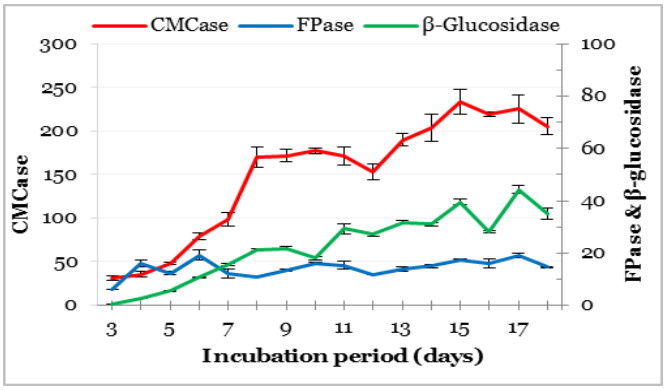

Figure 3: Time course study on cellulase production (IU/g) under SSF by *F. meliae* (values are Mean ± SEM)

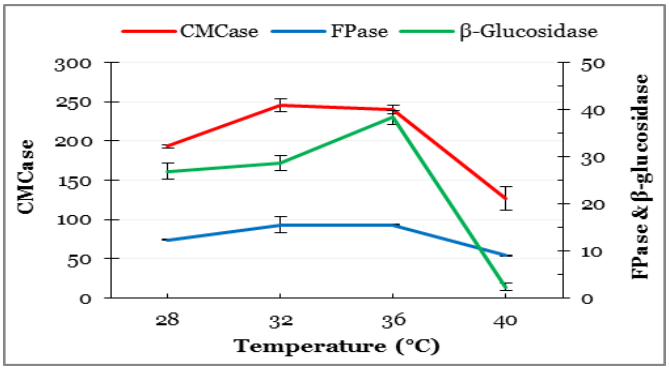

Figure 4: Effect of temperature on cellulase production (IU/g) under SSF by *F. meliae* (values are Mean ± SEM)

between 15–19 IU/g. β-glucosidase production gradually increased, reaching its maximum on day 17.

3.2. Effect of physiological factors on cellulase production under SSF

3.2.1 Temperature of the growth medium: A temperature range of 32–36 °C was found supportive for cellulase production. However, further increasing the temperature to 40 °C led to a sharp decline in enzyme yield.

3.2.2 pH of the growth medium: CMCase and β-glucosidase production were maximally at pH 4. However, increasing the pH from 4 to 6 and further to 8 did not substantially decrease their production. In contrast, FPase production remained consistent throughout the tested pH range of 4–8.

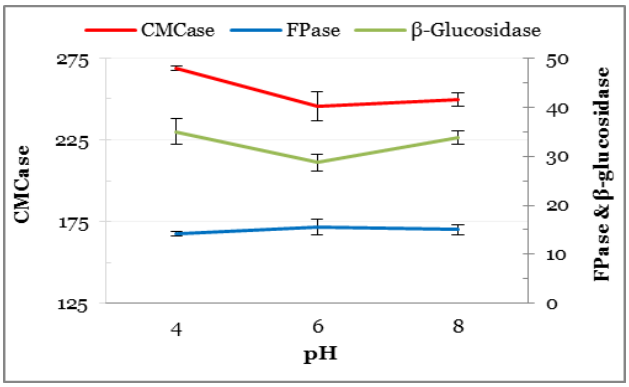

Figure 5: Effect of pH on cellulase production (IU/g) under SSF by *F. meliae* (values are Mean ± SEM)

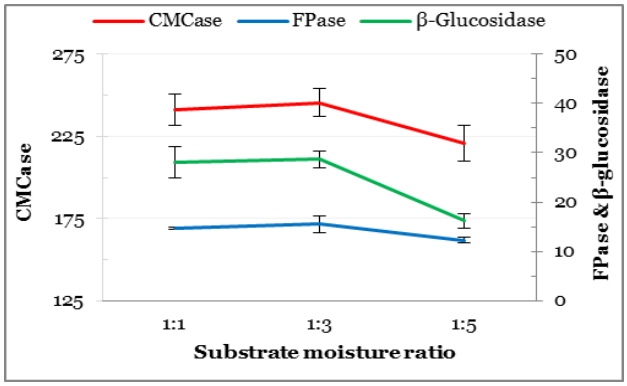

Figure 6: Effect of substrate moisture ratio on cellulase production (IU/g) under SSF by *F. meliae* (values are Mean± SEM)

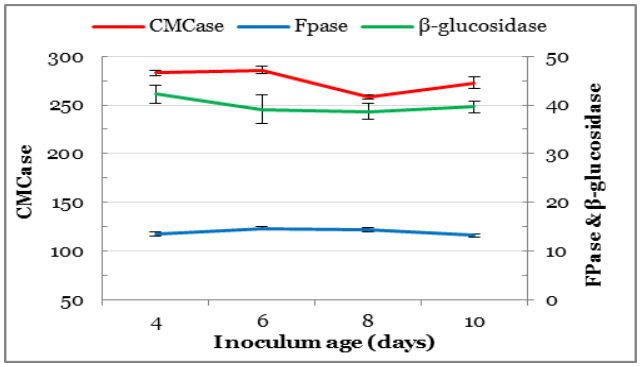

Figure 7: Effect of inoculum age on cellulase production (IU/g) under SSF by *F. meliae* (values are Mean ± SEM)

3.2.3 Substrate moisture ratio: At substrate moisture ratio of 1:1 and 1:3, the production of all three enzymes was statistically equal. However, their level declined at 1:5 substrate moisture ratio, with a decrease observed for CMCase and β-glucosidase.

3.2.4 Inoculum age: CMCase production declined slightly when inocula older than 6 days were used, whereas production of FPase and β-glucosidase remained consistent regardless of the age of inocula.

3.2.5 Inoculum dose: Increasing the inoculum dose from 1 to 7 discs did not significantly affect enzyme production (Figure 8).

3.2.6 Substrate quantity: CMCase and β-glucosidase production was maximal with 5 g of substrate, but their production declined sharply while increasing substrate quantity. The yield of FPase remained same regardless of increasing the substrate quantity from 5g to 20 g.

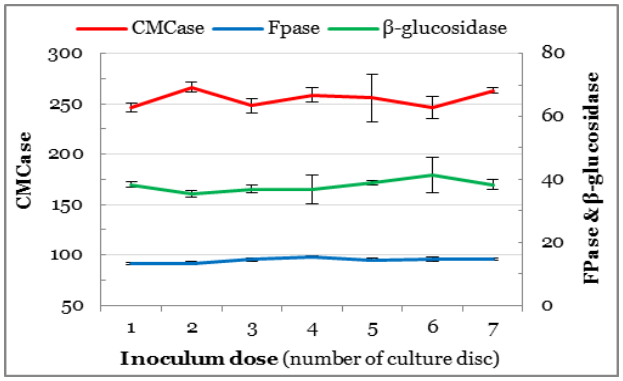

Figure 8: Effect of inoculum dose on cellulase production (IU/g) under SSF by *F. meliae* (values are Mean ± SEM)

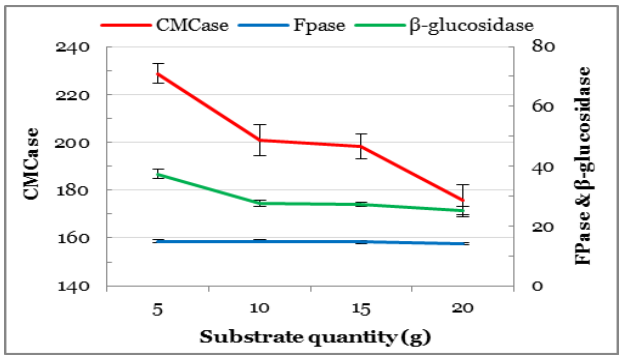

Figure 9: Effect of substrate quantity on cellulase production (IU/g) under SSF by *F. meliae* (values are Mean±SEM)

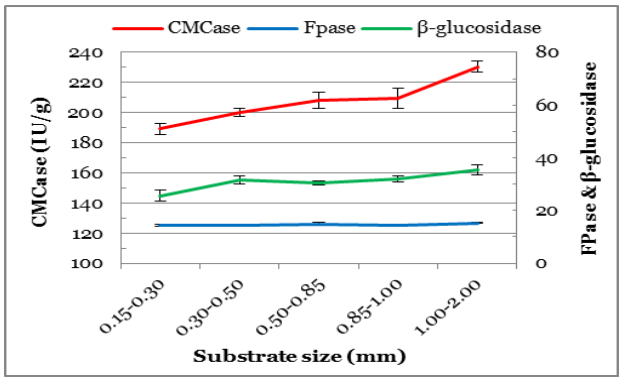

Figure 10: Effect of substrate size on cellulase production (IU/g) under SSF by *F. meliae* (values are Mean±SEM)

3.2.7. Substrate size: As size of the substrate increased, both CMCase and β-glucosidase production increased, while FPase activity remained constant. The maximum production of all three enzymes was recorded with the largest substrate size of 0.85–2.00 mm.

4. Discussion

4.1 Effect of time course on cellulase production

4.1.1 Under SmF: Incubation time has a direct effect on enzyme production to some extent (Gautam et al., 2011). The production of three enzymes began to rise from day 8 onwards and reached its peak on 10th day for CMCase and FPase, which is produced earlier compared to the β-glucosidase, reaching its maximum on the 19th day. The enzyme production gradually increased until reaching its peak and then started to decline from thereafter. Such activity pattern could be due to the gradual depletion of nutrients from the growth medium, which in turn changes the physiology of fungal culture and results in inactivation of the secretory machinery of enzymes (Nochure et al., 1993). Gautam et al. (2011) reported a 6 day incubation period as the most suitable duration for *Trichoderma viride*, while Kanakaraju et al. (2020) found day 5 to be suitable for *Aspergillus niger*. We also found that production of CMCase was higher than FPase, followed by β-glucosidase. Kanakaraju et al. (2020) and Belal et al. (2021) also reported similar results in Aspergillus niger where CMCase production was greater than FPase. However, Gautam et al. (2011) reported different results, with FPase being produced more, followed by β-glucosidase and then CMcase by *Trichoderma viride*.

4.1.2 Under SSF: The yield of cellulase in SSF was higher in comparison to SmF. This could be because SSF provides a natural environment for filamentous fungi to thrive, hence leading to larger fungal biomass production, resulting in higher yield of cellulases. The maximum production of CMCase, FPase, and β-glucosidase was obtained under SSF on the 17th day of incubation. CMCase was produced in the highest quantity, but contrary to SmF, β-glucosidase was produced more than FPase. Similar results were found by *Deswal et al. (2011)* in *Fomitopsis* sp., where CMCase was produced more than FPase and β-glucosidase, but their maximal production occurred on the 11th, 16th and 15th day, respectively. In this experiment, it was observed that as the incubation period increased, the production also increased up to a certain point. This is probably because with increasing incubation time, mycelial growth increases, which in turn provides higher enzyme production (Tu et al., 2007).

4.2 Effect of physiological factors on cellulase production under SSF

4.2.1 Temperature growth medium: The temperature of the growth medium is considered one of the most important variables in enzyme production in SSF (Bansal et al., 2012). In the present study, the highest production of CMCase, FPase and β-glucosidase was found in the temperature range between 32–36 °C. Iqbal et al. (2010) reported the maximum cellulase activity at 35 °C for *Trichoderma harzianum*, and Gilna and Khaleel (2011) for *Aspergillus fumigatus* at 32 °C, both falling within the temperature range applied in our experiment. This temperature range may be favorable for of mycelia growth and cellulase production, while lower temperature can hamper the uptake of nutrition by organisms, resulting in reduced metabolic activity (Oyeleke et al., 2012). Mycelia growth was observed to be very low at 40 °C compared to other temperature tested; hence, resulting in a sharp dip in production.

4.2.2 pH of growth medium: pH is also considered one of the influential factors affecting the mycelial growth, enzyme production and movements of various components through cell membrane (Kapoor et al., 2008). In this study, the highest production of both CMCase and β-glucosidase occurred at pH 4, whereas FPase production remained consistent throughout the pH range of 4–8. For cellulase production by microorganisms, a pH range of 4.0–6.0 has been reported as ideal (Tangnu et al., 1981). Prasetyo et al. (2010) also reported pH 4 as ideal for endoglucanase, but pH 5.5-6 for β-glucosidase for *Acremonium cellulolyticus*. Deswal et al. (2011) reported the optimum pH to be 5.5 for *Fomitopsis* sp. These findings suggest that the ideal pH conditions for maximum enzyme production vary among fungal species (Niranjane et al. 2007; Rabby et al., 2022).

4.2.3 Substrate moisture ratio: In SSF, moisture content in substrate plays an important role as it facilitates the solubility of the substrate, thus enabling better utilization by microorganisms for their growth. In the present study, a substrate moisture ratio of 1:1 to 1:3 was found to be optimum, providing a maximum production of all three enzymes. This result is in agreement with Dutt and Kumar (2014), who reported highest CMCase and FPase production at 1:3 substrate moisture ratio by *Apergillus flavus* and *A. niger*. Further increase in moisture content suppressed enzyme production. This occurred because high moisture content tends to reduce substrate surface area, decreases substrate porosity and restrict oxygen transfer (Bansal et al., 2012).

4.2.4 Inoculum age: Inoculum age influences mycelial colonization, which in turn can increase enzyme production (Bhargav et al., 2008). It has also been reported that inoculum age affects fungal morphology,

which correlates with enzyme production (Cui et al., 1998; Ferreira et al., 2009). We used inocula aged 4 to 10 days for cellulase production and found that CMCase production was slightly higher when younger incocula of 4-6 days of age were used, whereas the amount of β-glucosidase and FPase remained throughout consistent. Saini et al. (2017) observed that *Trichoderma reesei* produced high levels of CMCase and FPase with 4-day and 5-day-old inocula respectively. Higher cellulase production has been recorded in *Aspergillus hortai* with a 6-day-old culture (El-Hadi et al., 2014), *Penicillium oxalicum* with 7-day-old culture (Shah et al., 2015), *Aspergillus niger* with 5-day-old culture (Mandal and Ghosh, 2017).

4.2.5 Inoculum dose: Low inoculum dose results in a slower mycelial growth, poor substrate utilization, and thus lesser enzyme production. Conversely, high inoculum dose may also suppress enzyme production because of overcrowding of fungal mycelia, resulting in limited access to nutrition and oxygen (Saravanan et al., 2012). In the present study, an increase in inoculum dose from 1 to 7 discs (each 8 mm in size) per 5 g substrate did not influence in any way. There was just a slightly higher yield of all the three enzymes with 4 discs inoculation. The results indicate that mycelia of *F. meliae* perhaps colonize faster on wheat bran. Bansal et al. (2012) reported an optimal dose of 5 discs (each 7 mm in size) for *Aspergillus niger*, whereas Saini et al. (2017) reported 10 discs for *Trichoderma reesei* for enhanced cellulase production.

4.2.6 Substrate quantity: The yield of CMCase and β-glucosidase was maximal when 5 g of substrate was used, while FPase production was not affected by substrate quantity. Higher cellulase production during SSF with 5g substrate has been reported for *Aspergillus niger* on banana peel (Mandal and Ghosh, 2017*), Coprinopsis cinerea* on sugarcane baggase (Maan et al., 2016) and *Penicillium notatum* on cabbage waste (Das and Ghosh, 2009). An optimal amount of substrate is required to maintain a proper thickness layer, ensuring adequate substrate porosity and gas exchange (Montoya et al., 2021) which influences cellulase production and initial hydrolysis rate of the enzyme (Iqbal et al., 2010).

4.2.7 Substrate size: It is reported that an adequate particle size of the substrate provides better surface area and requisite aeration for microbial metabolism, growth and enzyme production (Assamoi et al., 2008; Maan et al., 2016). Nevertheless, optimum substrate size varies according to the nature of the substrate used. In this study, we found that as the substrate size increased, the cellulase production also increased, reaching its maximum with wheat bran particle sizes ranging 0.85-2.00 mm. For cellulase production under SSF, a particle size of 180 µm for banana agrowaste with *Penicillium oxalicum* (Shah et al., 2015), and a particle size of 300 µm for sugarcane baggase with *Coprinopsis cinerea* (Maan et al., 2016) has been reported as appropriate.

5. Conclusion

The results of the present study indicate *F. meliae* as a potential cellulase producing fungus, performing better in SSF than in SmF. It produced more CMCase than β-glucosidase and FPase under SSF. Further optimization of physical parameters enhanced CMCase production, while FPase and β-glucosidase production remained almost constant. Cellulase production by this brown rot fungus may be further enhanced by optimizing various nutritional parameters of the growth medium, either through OFAT or through statistical methods such as Plackett-Burman Design (PBD) and Response surface methodology (RSM). These are less time-consuming and also take into account the interaction between various parameters.

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Author(s) contribution

Gegam Jini: Conceptualization, Experimentation, Statistical analysis, and Writing draft manuscript. Rajiv Kumar Singh: Conceptualization, Supervision, Review of the manuscript.

Conflict of interest

Authors declare have no conflict of interests.

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